IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#8

In Re Application of:

COX et al.

Serial No.: 09/897,844 Art Unit: Unassigned

Filing Date: July 2, 2001 Examiner: Unassigned

Title: REGULATION OF ENDOGENOUS GENE EXPRESSION USING ZINC FINGER PROTEINS

DECLARATION UNDER 37 C.F.R. § 1.132

I, Frank Giordano, declare as follows:

1. I am presently an Assistant Professor in the Department of Medicine, Section of Cardiovascular Medicine at Yale University and have held this position since 1999. Prior to joining the faculty at Yale, I was an Assistant Professor in the Department of Medicine, Cardiology Section, at the University of California, San Diego. I received my M.D. from Loyola University Stritch School of Medicine in 1986. I completed my internship and residence in Internal Medicine at Loyola University and also completed two fellowships at the University of California, San Diego. A copy of my Curriculum Vitae (Exhibit A) is attached hereto. I have considerable experience in the area of interventional cardiology including using gene therapy techniques in animal model systems and in clinical trials to assess angiogenesis and vessel formation.

2. Experiments conducted in my laboratory have demonstrated that ZFPs can regulate expression levels of endogenous VEGF *in vivo*. Additionally, using several different accepted live animal model systems for angiogenesis and wound healing, the results from these studies show that engineered ZFPs can be utilized to modulate vessel formation and wound healing via regulation of one or more VEGF genes.

3. Plasmids encoding ZFPs targeted to both the rat and mouse VEGF genes were supplied by Dr. Casey C. Case, Sangamo Biosciences, who asked us to perform certain experiments in animal model systems. ZFP-encoding plasmids provided by Sangamo Biosciences were used, in some experiments, without modification. In addition, using the Sangamo Biosciences polynucleotides as starting material, we prepared ZFP-encoding adenovirus vectors, as described in Exhibit C, entitled

"Experimental Protocols." ZFP encoding plasmids and ZFP-encoding adenovirus vectors were administered to live animals, and the animals were assayed for VEGF expression, vascularization and wound healing, as described in Exhibit C.

4. Our results show that VEGF expression was enhanced *in vivo* in both skeletal and cardiac muscle upon injection of plasmids encoding VEGF-targeted ZFPs. Figure 1 of Exhibit B shows the results of an experiment in which a plasmid encoding the MVG10A/8A ZFP, fused to a VP16 activation domain, was injected into mouse quadriceps muscle, resulting in increased VEGF expression in the injected skeletal muscle. VEGF expression was also enhanced in murine cardiac muscle following administration of a ZFP/VP16-encoding plasmid. In addition, ZFP/VP16 fusions targeted to the rat VEGF gene enhanced expression of VEGF in rats. For example, Figure 2 of Exhibit B shows levels of VEGF-A protein expression in Sprague-Dawley rats following injection of plasmids encoding fusions of VP16 to VOP 30A, VOP 32B, BV012A and BV014A; compared to control plasmid injections. The BV012A, BV014A, and VOP 32B fusions increased VEGF expression between 5- and 10-fold. The internal actin standard indicates that enhancement of VEGF expression in these tissues was not due to injection *per se*. These studies demonstrate that plasmid constructs encoding ZFPs regulate gene expression *in vivo*.

5. VEGF expression was also enhanced *in vivo* in both skeletal and cardiac muscle upon administration of adenovirus vectors encoding ZFPs. Figure 3 of Exhibit B shows that adenovirus-mediated expression of a VOP30A/VP16 fusion gene resulted in a marked induction of the VEGF-A gene *in vivo* in the hindlimb adductor muscle of CD-1 mice, compared to muscles that had been injected with an adenovirus expressing green fluorescent protein (GFP). Duplicate samples are shown.

6. Thus, using the polynucleotides encoding VEGF-targeted ZFPs provided by Sangamo Biosciences, we demonstrated that various gene delivery vectors encoding these ZFPs were able to regulate expression of VEGF *in vivo*.

7. Using several different accepted model systems for angiogenesis and wound healing, our investigations also show that VEGF-targeted ZFPs can be utilized to modulate angiogenesis and thus affect a wide variety of *in vivo* conditions that are correlated with blood flow and blood delivery. More specifically, our results demonstrate that angiogenesis and related processes can be regulated by introducing gene delivery vehicles (*e.g.*, plasmids, viral constructs, *etc.*) encoding ZFPs which modulate expression of one or more VEGF genes *in vivo*. Increased vessel counts, enhanced wound re-epithelization and accelerated wound healing were demonstrated in standard

animal model systems using established histological and immunohistochemical methods. (See Exhibit C for a description of methods).

8. An established model system for analysis of angiogenesis is evaluation of vessel formation in the murine ear. Using this system, the number of blood vessels, as determined using immunostaining techniques (see Experimental Protocols, Exhibit C), at either three or six days after injection of a mouse ear with an adenovirus encoding a ZFP (VOP32B or VOP30A)-VP16 fusion was evaluated. The results for VP32B are shown in Figure 4, in which the dark bars represent vessel counts in ears injected with a ZFP-encoding adenovirus, and lighter bars show vessel counts in ears injected with a green fluorescent protein-encoding adenovirus. Similar results were obtained with VOP 30A. These results indicate increased vascularity in mouse ear following injection with ZFP-VP16-adenovirus constructs, compared to control injected ears.

9. VEGF-regulating ZFPs also augment wound healing (*e.g.*, re-epithelization, keratinocyte ingrowth) in a well-established cutaneous wound healing model (see Exhibit C for details on experimental procedures). Bilateral cutaneous wounds were created in the backs of CD-1 mice by excision of a 5 mm circle of skin using a punch bioptome. At the time of wounding, adenovirus encoding a MVG/VP16 fusion was applied topically to one of the wounds. The contralateral wound was subjected to topical application of an adenovirus encoding green fluorescent protein. Five days after treatment, the wounds were excised, fixed, embedded in paraffin, and histological sections were prepared and examined. The distance between leading edges of keratinocyte ingrowth were measured in the sections and the results are shown in Figure 5 of Exhibit B. These results indicate that there is a significant reduction in distance (*i.e.*, faster ingrowth) after treatment with the VEGF-targeted ZFP (dark bar), compared to treatment with the GFP control (lighter bar). These results were also confirmed by observation of the ingrowth of tissue beneath the blood clot covering the wound. While significant ingrowth was apparent in ZFP-treated wounds (Figure 6 of Exhibit B, panels B, D and F), no ingrowing tissue was seen beneath the clot in the control-injected tissue. (Figure 7 of Exhibit B, panels A, C and E). Thus, *in vivo* activation of endogenous VEGF by targeted ZFP molecules results in faster wound healing as evidenced by increased re-epithelization and more rapid keratinocyte ingrowth.

10. ZFP-mediated activation of VEGF expression also stimulates angiogenesis in the mouse cutaneous wound healing model system. Cutaneous wounds treated as described in paragraph 9 were fixed, sectioned and immunostained with an

antibody against an endothelial cell-specific lectin. Using antibody staining as an indication of vascularirty, vessel counts were performed on digitally captured images of sections of ZFP-treated and control wounds. As shown in Figure 7 of Exhibit B, increased vascularity is observed in wounds that had been treated with an MVG/VP16 expressing adenovirus (dark bar), compared to control wounds that had been treated with a green fluorescent protein-encoding adenovirus (lighter bar). These results are consistent with histological results, in showing that VEGF-targeted ZFPs, fused to an activating domain, increase angiogenesis *in vivo*.

11. In light of these results, I conclude that engineered ZFPs can be used to modulate expression of endogenous genes in living animals. Modulation of endogenous gene expression by engineered ZFPs can be accomplished using a variety of different zinc finger proteins and a variety of different administration modalities (*e.g.*, injection and/or topical administration into skeletal muscle, cardiac muscle and/or ear tissue using plasmid, viral or other vectors). Further, the studies reported herein indicate that modulation of endogenous gene expression by engineered ZFPs can affect conditions associated with expression of the target gene; for example, modulation of VEGF expression affects the processes of angiogenesis and wound healing. Because ZFPs can be designed and/or selected to bind to any predetermined sequence, methods similar to those described in this declaration are equally applicable to any endogenous gene of interest.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 11/2/01 Signature: Frank Giordano

Frank Giordano, M.D.

EXHIBIT A

Frank J. Giordano, MD

PERSONAL RECORD

DATE/PLACE OF BIRTH: March 15, 1957; Chicago, Illinois

SPOUSE: Catherine Leadon Giordano, MD

ADDRESS:
Laboratory: Boyer Center for Molecular Medicine; Rm 336C
Yale University School of Medicine
295 Congress
New Haven, CT 06510
(203) 785-7631 (office)
(203) 785-7967 (lab)
(203) 737-2286 (FAX)
e-mail: Frank.Giordano@yale.edu

Home: 412 Bartlett Drive.
Madison, CT 06443
(203) 421-0696

PROFESSIONAL EMPLOYMENT

1999-present
- Assistant Professor; Dept. of Medicine; Section of Cardiovascular Medicine; Yale University School of Medicine
- Director of Cardiovascular Gene Therapy Program
- Associate Director, Vector Core

1995-1999
- Assistant Professor of Medicine; Cardiology Section; University of California San Diego
- Instructor; Cardiology Section; University of California San Diego

FELLOWSHIP

7/94-6/95
- Interventional Cardiology; (Maurice Buchbinder); University of California San Diego

7/92-6/94
- Post-doctoral fellowship/Molecular Biology; University of California San Diego

7/90-6/92
- Clinical cardiology; University of California San Diego

INTERNSHIP/RESIDENCY

6/89-6/90
- Chief Resident-Internal Medicine; Loyola University, Maywood IL.

6/86-6/89
- Resident-Internal Medicine; Loyola University; (Rolf Gunnar, Chairman)

EDUCATION

1982-1986
- Loyola University Stritch School of Medicine; MD, Cum Laude

3/77-12/80
- University of Illinois; BS Biology

8/75-12/76
- Indiana University

AWARDS / HONORS
- AOA Medical Honor Society
- M.D. Cum Laude
- National AHA Scientist Development Award; 1998-2002
- Parke-Davis ARA Research Award; 1999-2001
- Patterson Foundation Award; 1999-2000
- UCSD Attending Teaching Award; 1997
- Schulman Prize for Cardiovascular Research; UCSD; 1995

- Smith Kline Beachman Young Investigators Award; 1992
- NIH NRSA; 1992-1994

CERTIFICATION

1993
- Diplomate Cardiovascular Disease Subspecialties; American Board of Internal Medicine

1989
- Diplomate American Board of Internal Medicine

LICENSURE

1998-present
- Physician and Surgeon; State of Connecticut

1990-present
- Physician and Surgeon; State of California

1986-1991
- Physician and Surgeon; State of Illinois

GRANTS/FUNDING (Principal Investigator; Active)

2001-2006
- *Molecular Egress in Gene Delivery; NIH RO1*

2000-2005

- *Hypoxia-induced transcription in cardiovascular function; NIH-RO1*

1998-2002
- *The Role of VEGF in Cardiac Development, Coronary Vascularization and Adaptation to Cardiac Hypertrophy; National AHA SDG Grant*

2000-2002
- *Regulation of angiogenesis by engineered zinc finger proteins; Edwards Lifesciences*

1999-2001
- *HIF-1 α–mediated hypoxic signaling in angiogenesis and vascular remodeling; Parke-Davis ARA Award*

1999-2001
- *Molecular Imaging of Angiogenesis; Dupont*

GRANTS/FUNDING (Collaborating Investigator; Active)

2001-2006
- *Molecular Imaging of Angiogenesis; NIH RO1; P.I. Albert Sinusas*

GRANTS/FUNDING (previously active)

1999-2000
- *The role of leptin in myocardial angiogenesis and ventricular remodeling; Patterson Foundation Award*

1997-1998
- *Intracoronary and Intravenous Vascular Endothelial Growth Factor for Induction of Myocardial Angiogenesis; Genentech*

1997-1998
- *Vascular Endothelial Growth Factor Dosing Toxicology Study; Genentech;*

1996-1997
- *Myocardial Delivery of the SERCA2 Gene- Co-investigator; Genzyme Corporation;*

1995-2000
- *Alterations in the Expression of Membrane Calcium Pump Genes in Cardiac Hypertrophy;* (NIH RO1; Co-Investigator)

1995-1998
- *Modulation of Post-Infarction Ventricular Remodelling; (Collaborator; NIH RO1)*

1996-1997
- *Electroporation-mediated Gene Transfer - Co-investigator; Genetronics Inc.;*

1992-1994
- *Regulation of SERCA2 Expression in Cardiac Myocytes; NIH-NRSA*

1993

- *Potential protective effects of T3 during experimental ischemia; SmithKline-Beecham*

PENDING GRANTS:

- *Imaging DTH, IFN-γ Responses and GA in Human Arteries; (Project in NIH PO-1; Co-Investigator)*
- *Molecular and Non-Invasive Imaging Approaches for the Characterization of Transplant Vasculopathy; (Project in* NIH CRC Renewal)
- *System for Targeted Delivery of Therapeutic Agents* (Involves the use of ultrasound activated microbubbles for selective delivery of therapeutic genes and peptides to the heart) (collaborator); Submitted as a Bioengineering Research Partnership Application to NIH

CLINICAL SKILLS/EXPERIENCE

INTERVENTIONAL/INVASIVE CARDIOLOGY

- Extensive experience in high risk and multi-vessel coronary revascularization (rotational atherectomy, directional coronary atherectomy, endovascular stent deployment, balloon angioplasty, coronary laser, balloon valvuloplasty, endomyocardial biopsy, intravascular ultrasound, intravascular doppler and flow wire).
- Experienced in pulmonary angiography in the setting of marked pulmonary hypertension.

CLINICAL TRIALS

VIVA; A Phase II Double Blinded Placebo Controlled Study to Assess the Efficacy of rhVEGF administration in Subjects with Viable but Underperfused Myocardium; Principle Investigator UCSD

LIMIT Trial; Phase II study of anti-CD18 antibody in patients with acute MI; Principle investigator UCSD

A Phase I, Open-Label Study To Assess the Safety, Tolerability, and Pharmacokinetics of Intracoronary Administration of rhVEGF in Subjects with Viable but Underperfused Myocardium; Principle Investigator UCSD; ; Also involved in preclinical testing and study design.

A Phase I, Open-Label Study To Assess the Safety, Tolerability, and Pharmacokinetics of Intravenous Administration of rhVEGF in Subjects with Viable but Underperfused Myocardium; Principle Investigator UCSD; Also involved in preclinical testing and study design.

Molecular and Non-Invasive Imaging Approaches for the Characterization of Transplant Vasculopathy; Principle Investigator; Single site study, Yale University; ongoing.

RESEARCH FOCUS

RESEARCH PROJECTS ONGOING OR COMPLETED DURING THE LAST 3 YEARS

- Oxygen sensing/signaling and transcriptional regulation of vascularization, metabolism and cardiac function: role of hypoxia inducible factor HIF-1a signaling. (Involves investigation of the role of the hypoxia-inducible factor HIF-1α in mice with cardiac specific HIF-1α gene deletions); NIH RO1 funded.

- "Molecular Egress in Gene Delivery" (Involves investigation of the role of peptide sequences in directing transendothelial and intracellular trafficking of engineered gene therapy vectors); NIH RO1 funded.

- "HIF-1alpha-mediated signaling in angiogenesis and vascular remodeling" (Involves investigation of role of the hypoxia-induced transcription factor HIF-1α in endothelial and smooth muscle cell development and function in endothelial and smooth muscle specific knockout mice); NIH and Parke-Davis ARA Award funded.

- "The role of VEGF in cardiac development, coronary vascularization, and adaptation to cardiac hypertrophy" (The major goals of this project are to study the role of cardiac myocyte specific VEGF production on development and response to physiologic stress); AHA funded.

- "System for Targeted Delivery of Therapeutic Agents" (Involves the use of ultrasound activated microbubbles for selective delivery of therapeutic genes and peptides to the heart); Collaborative funding via NIH (Bioengineering Research Partnership Application) pending.

- "The role of Leptin in Myocardial Angiogenesis and Ventricular Remodeling" (This project studies cardiac leptin expression and effects on angiogenesis and wound healing in a model of myocardial infaction); Funded by Patterson Trust Foundation

- Molecular mechanisms defining AAV transduction efficiency in the vasculature and myocardium (involves study of the mechanisms defining internalization and double stranded DNA formation in host cells during AAV transduction).

- Cardiomyocyte calcium handling – (Involves the role of the sarcoplasmic reticulum calcium ATPase (SERCA2) and the physiologic effects of overexpression (transgenic mouse and adenovirus gene transfer based strategies) and reduced expression of this membrane pump. Clinical modulation of this system to treat heart failure is a focus of our gene therapy efforts.

- "The use of engineered Zinc Finger Proteins to modulate angiogenesis" (Involves the design and in vivo delivery of novel zinc finger protein constructs that modulate activity of endogenous genes);

TRANSLATIONAL RESEARCH

- Developed an intracoronary approach for gene delivery to the heart and developed a strategy for gene therapy based therapeutic myocardial angiogenesis. This effort led to

inventorship and granting of a patent that formed the basis of a new biotech company (Collateral Therapeutics) in which I do not participate.

- Developed a gene therapy based approach for augmentation of myocardial contractile function based on modulation of dynamic calcium handling. This effort led to inventorship and issue of a U.S. patent.

- Developed a gene therapy based approach for myocardial protection based on modulation of heat shock protein expression. Inventorship and issue of a U.S. patent.

- Contributed to the development of a catheter based system for electroporation based gene delivery. Inventorship; patent pending.

- Director of Cardiovascular Gene Therapy Program and Associate Director of the Vector Core; Current focus on macromolecular transport and vector targeting, development of alternative non-viral gene delivery vehicles. Major focus on the use of engineered Zinc Finger Proteins to modulate gene expression in the cardiovascular system. Clinical trials in pre-clinical and planning stages.

- Modulation of stem cell gene program to effect cardiac differentiation and myocardial repair.

- Stimulated Therpeutic angiogenesis: PI, preclinical studies of hemodynamic effects and angiogenic effects of in vivo rhVEGF165; PI for three clinical trials of rhVEGF165 protein in patients with ischemic heart disease.

- PI, study of the role of leptin in the cardiovascular system and its regulation after acute MI. Correlated expression levels in patients with hypoxic gene regulation in cardiac myocytes and fibroblasts and in a rat infarct model.

- Collaborator; Cardiac Nuclear Imaging group on the development of non-invasive targeted imaging strategies for the detection of angiogenesis.

- Collaborator; Bioengineering Dept. on the development of microbubble technology for gene delivery to the heart.

Professional Organizations

- American Heart Association
- American College of Cardiology
- American Federation of Clinical Researchers
- American Association for the Advancement of Science
- American College of Physicians; (Organized 1989 Illinois Associates Annual Scientific Session)

SELECTED INVITED PRESENTATIONS

FDA; Biomed Research Foundation: Delivery of Angiogenic Factors: Gene vs.Protein; 2000

European Heart Association International Congress (Barcelona, 1999) "*Efficacy of Angiogenic Protein Delivery for Therapeutic Angiogenesis*"

Brigham and Womens Vascular Biology Seminar Series; "*Cardiac gene targeting of VEGF and HIF-1α"*

Beth Israel/Harvard; "*Approaches to therapeutic cardiovascular gene delivery" Brigham and Women's Hospital; "Molecular Approaches to Stimulated Therapeutic Angiogenesis*"

European Heart Association International Congress (Stockholm) "*Molecular Therapies for Ischemic Heart Disease*"

University of Toronto (Division of Cardiology) "*Therapeutic Myocardial Angiogenesis" and "Therapeutic Cardiovascular Gene Transfer*"

Second Annual International Local Drug Delivery Seminar at MIT "*Therapeutic Cardiovascular Gene Transfer*"

Duke University - "*Myocardial gene delivery and stimulated angiogenesis*"

NIH - "*Stimulated Angiogenesis by Intracoronary Gene Delivery*"

Northwestern University - "*FGF-5 Gene Delivery for Myocardial Angiogenesis*"

LaJolla Cancer Institute/ Burnham Institute "*Stimulated Angiogenesis by Myocardial Gene Transfer*"

PATENTS

In vivo Gene Delivery for Ischemic Heart Disease (*Gene delivery for modulation of heat shock proteins in the heart*)- Granted

In vivo Gene Delivery for Ischemic Heart Disease (*Gene delivery based stimulation of angiogenesis*)- Granted

In vivo Gene Delivery for Treatment of Heart Failure (Gene delivery for modulation of calcium handling in the myocardium)- Granted

Electroporation Mediated Delivery of Bioactive Peptides - Pending

Induction of cardiac differentiation for myocardial repair - Pending

TEACHING EXPERIENCE AND TRAINEES:

TRAINING GRANT PARTICIPATION

Vascular Biology Training Grant; Yale University (P.I. Jeffrey Bender);

Molecular Cardiobiology (Kenneth Chien-PI, UCSD)

CURRENT TRAINEES; BASIC RESEARCH

1. Jennifer Yeh, M.D.; Postdoctoral Fellow studying oxygen sensing/signaling in smooth muscle cells.

2. Yan Huang, Ph.D.; Postdoctoral scientist studying the role of the hypoxia responsive transcription factor HIF-1α in knockout mice.

3. Dawn Abbott, M.D.; Postdoctoral Fellow studying the behavior of human stem cells in rodent models of myocardial injury.

4. Marie-Jeanne Montisci, Ph.D.; Postdoctoral scientist/polymer chemist, studying peptide and macromolecular internalization pathways in cardiac myocytes and stem cells.

5. Wayne Huang, M.D.; Postdoctoral fellow using in vivo phage display to define pathways of macromolecular transport across continuous endothelia.

6. David Lao, B.S.; Yale medical student who is studying transcriptional and post-translational regulation of HIF-1α and EPAS1 in endothelial cells.

PAST TRAINEES; BASIC RESEARCH:

1. David Friedmann, M.D.; Yale medical student who spent 7 months in my lab studying AAV transduction kinetics in endothelial cells and constructing AAV vectors encoding Kallikrein (work submitted, and in preparation).

2. David Clark, M.D.; Yale interventional cardiology fellow who completed a combined clinical and basic research effort in my lab documenting the expression and hypoxic induction of leptin and leptin receptors in cardiac myocytes and fibroblasts, and documenting a marked rise in serum leptin levels with loss of diurnal variation in patients after myocardial infarction; (work submitted).

3. Jesse James, B.S.; Undergraduate who spent a summer in my lab as part of a Yale based program to facilitate the development of minority physician-scientists. He is now in medical school at Yale and his work is being completed for submission.

CLINICAL TEACHING (current):

Director of interventional cardiology fellowship outpatient clinical rotation, Yale University.

Responsible for instruction of cardiology fellows in all aspects of diagnostic and interventional cardiac catheterization; Responsible for instruction of students, residents and fellows in cardiac critical care unit.

PUBLICATIONS

1. Ron Hartong, Francisco Villareal, **Frank J. Giordano**, Noel Kim, Marja Brinker and Wolfgang Dillmann; Phorbol Myristate Acetate Induced Hypertrophy of Neonatal Rat Cardiac Myocytes Is Associated With Decreased Sarcoplasmic Reticulum Calcium ATPase (SERCA2) Gene Transcription. *JMCC* 1996, V28:2467-2477

2. Ruben Mestril, **Frank J. Giordano**, Andre G. Conde, Wolfgang H. Dillmann; Adenovirus Mediated Gene Transfer of Heat Shock Protein 70 (HSP70i) Protects Against Simulated Ischemia; *JMCC* 1996, V28:2351-2358

3. Francisco Villareal, Ann Lee, Wolfgang H. Dillmann and **Frank J. Giordano**; Adenovirus Mediated Overexpression of Human Transforming Growth Factor-β1 in Rat Cardiac Fibroblasts, Myocytes and Smooth Muscle Cells; *JMCC* 1996, V28 N4:735-742

4. **Frank J. Giordano**, Peipei Ping, M. Dan McKirnan, Shiro Nozaki, Anthony N. DeMaria, Wolfgang H. Dillmann, Odile Mathieu-Costello and H. Kirk Hammond. Intracoronary Gene Transfer of Fibroblast Growth Factor-5 Increases Blood Flow and Contractile Function In An Ischemic Region of the Heart; *Nature Medicine*, 1996, May, V2 N5:534-539

5. Chan-Yi Lu, **Frank J. Giordano**, Kimberlyn Rogers and Abraham Rothman; Adenovirus Mediated Increase of Exogenous and Inhibition of Endogenous Fos-B Gene Expression in Cultured Pulmonary Artery Smooth Muscle Cells; *JMCC.* 1996;28:1703-1713

6. Huaping He, **Frank J. Giordano**, M. Richard Sayen, Pat M. McDonnough, Howard Rockman, Randa Hilal-Dandan, Wolfgang H. Dillmann. Overexpression of Sarcoplasmic Reticulum Calcium ATPase in Transgenic Mice Accelerates Calcium Uptake by SR and Cardiac Relaxation; *J Clin. Invest.*, 1997;100:380-389

7. **Frank J. Giordano**, Huaping He, Pat M. McDonnough, Randa Hilal-Dandan, Marcus Meyer, M. Richard Sayen and Wolfgang H. Dillmann.Adenovirus Mediated Reconstitution and Overexpression of Sarcoplasmic Reticulum Calcium ATPase in Rat Cardiomyocytes; Circulation, 1997;96:400-403

8. Thistlewaite P, Tarazi RY, **Giordano FJ**, Jamieson SW. Surgical Management of Spontaneous Left Main Coronary Artery Dissection Annals of Thoracic Surgery, 1998;66:258-260

9. Chan-Yi Lu, **Frank J Giordano**, Kimberlyn Rogers and Abraham Rothman Antisense fosB RNA inhibits thrombin-induced hypertrophy in cultured pulmonary arterial smooth muscle cells; . Circulation, 1998;98:596-603

10. **Giordano FJ**. Thrombolysis or Primary PTCA: Which is best in acute myocardial infarction?; Topics in Emergency Medicine 1998;

11. Meyer M, Bluhm WF, He H, Post S, **Giordano FJ**, Lew W, Kranius EG, Dillmann WH. Phospholamban-to-SERCA2 ratio controls the force-frequency relationship. *AJP;Heart;* 1999;**276:**H779-H785

12. Hellerbrand C, Stefanovic B, **Giordano F**, Brenner DA. The role of TGFβ1 in initiating hepatic stellate cell activation in vivo. *Journal of Hepatology;* 1999;**30**:77-87

13. Dunn KC, Marmorstein AD, Bonilha VL, Rodriguez-Boulan E, **Giordano FJ**, Hjelmeland LM. Use of the ARPE-19 Cell Line as a Model Of RPE Polarity: Basolateral Secretion of FGF5. *IVOS;* 1998;**39**:2744-2749

14. Tsimikas S. **Giordano FJ**. Tarazi RY. Beyer RW. Spontaneous coronary artery dissection in patients with renal transplantation. Journal of Invasive Cardiology. 11(5):316-321, 1999 May

15. **Giordano FJ**. Angiogenesis: Mechanisms, modulation, and targeted imaging. [Article] Journal of Nuclear Cardiology. 6(6):664-671, 1999 Nov-Dec

16. Hendel RC, Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, **Giordano FJ**, Simons M, Bonow RO. Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. *Circulation* 2000 Jan 18;101(2):118-21

17. Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, **Giordano FJ**, Simons M, Losordo DW, Hendel RC, Bonow RO, McCluskey ER. Initial Results of Intracoronary Administration of Recombinant Human Vascular Endothelial Growth Factor (rhVEGF); (In Press).

18. Grunstein J, Masbad JJ, Hickey R, **Giordano FJ**, Johnson RS. Isoforms of vascular endothelial growth factor act in a coordinate fashion to recruit and expand vascular beds. Molecular & Cellular Biology. 2000, 20(19):7282-91

19. **Giordano FJ**, Johnson RS. Angiogenesis: the role of the microenvironment in flipping the switch; Current Opinions in Genetics and Development; 2001, 11:35-40

20. **Giordano FJ**, Gerber HP, Williams S, Bruggen NV, Bunting S, Ruiz-Lozano P, Nath AK, Huang Y, Hickey R, Chien KR, Ferrara N. A Cardiac Myocyte VEGF Paracrine Pathway is Required to Maintain Cardiac Function. *PNAS.* 2001, **98**:5780-5785

21. Bale TL*, **Giordano FJ*** Hickey R, Huang Y, Nath AK, Peterson KL, Vale WW, , Lee KF. Corticotropin-releasing factor receptor-2 deficient mice are hypervascularized: A novel role for CRFR2 as an angiogenesis inhibitor. (In Revision; *Nature*; *equal contribution)

22. Huang Y, Rhagu Katragadda, Nath AK, Hickey R, Abraham Rothman, Chan-Yi Lu, **Frank J Giordano**; $p15^{INK4B}$ gene expression induces apoptosis in vascular smooth muscle cells and resistance to apoptosis in skeletal myoblasts; (submitted)

23. **Giordano FJ**, Ross Jr J, Peterson KL, Dalton N, McCluskey T, Zionchek T, McKirnan MD; Infusion of VEGF by Intracoronary or Intravenous Route Ameliorates Ischemia Induced Contractile Abnormalities in the Pig; (submitted)

24. Clark D., Pfau S, Nath A.K., Clemens M., Setaro J., Brennan J., Cabin H, Sierra-Honigmann R, **Giordano FJ**. Elevated serum leptin levels in patients with acute myocardial infarction. (Submitted)

25. Ikeda Y, Gu Y, Oh S.S., Hoshijima M, **Giordano FJ**, Chen J, Nigro V, Peterson KL, Chien KR, Ross Jr. J.In Vivo Cardiac Gene Transfer of a Structural Protein Gene: Use of Hypothermic Cardiac Arrest in the Hamster. (submitted)

26. Nath A.K., Huang Y, Hickey R, Johnson RS, **Giordano FJ**. The role of HIF-1α signaling in the vascular proliferative response. (submitted)

27. Van Bruggen N, Gerber H.P., **Giordano F.J.**, Lozano P. Ruiz, Ryan AM, Sherman D, Chu M, Williams S, Chien KR Ferrara N. Magnetic Resonance Imaging of mice to assess heart function. (submitted)

28. Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, **Giordano FJ**, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson M, Fine J, Bajamonde A, McCluskey ER. The VIVA Trial: Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis. (submitted)

29. Toman J, Huang W, Montisci MJ, Huang Y, Hickey R, **Giordano FJ**. Specific peptide ligands mediated cardiac targeting in vitro and in vivo. (in preparation)

30. Montisci MJ, Huang W, Toman J, Huang Y, Hickey R, Nath AK, **Giordano F.J.** Specific peptide ligands that direct macromolecular transcytosis. (in preparation)

31. Simons M, **Giordano FJ**, Henry TD, Powell JS, Azrin MA, Kereiakes DJ, Annex B, Miller JM, Gibson CM, Lewin HC, Berman DS, McCluskey ER. Intravenous administration of recombinant human vascular endothelial growth factor (rhVEGF) in patients with ischemic heart disease; (In preparation)

32. Eppler SM, McCluskey ER, Bloedow DC, Henry TD, Simons M, **Giordano FJ**, Zioncheck TF. Blood pressure changes and pharmacokinetics following vascular endothelial growth factor (rhVEGF) infusion in humans; (In preparation)

33. **Giordano FJ**, Huang Y, Hickey R, Liu N, Nath AK, Johnson R. A critical role for basal cardiac HIF-1α expression in maintaining cardiac function and defining cardiac energetics. (in preparation)

EXTRAMURAL ADVISORY

Scientific Advisory Board: Genzyme
Consultant: Edwards Life Sciences
Sapien Inc.
Guidant Corporation

COMMITTEES (CURRENT ONLY)

Yale Residency selection committee
Yale Human Gene Therapy committee
Yale cardiology fellowship selection committee

EXHIBIT B

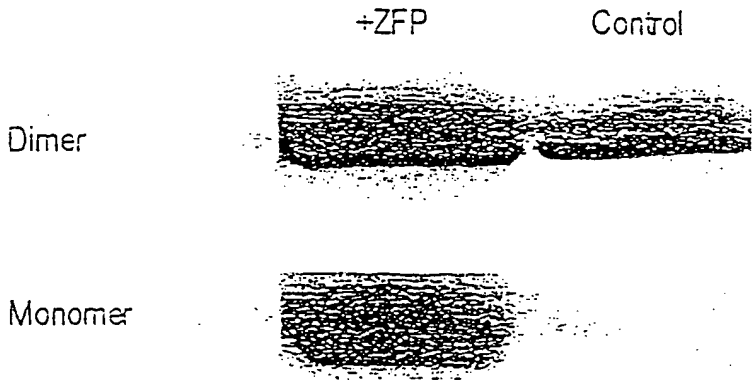


FIG 1

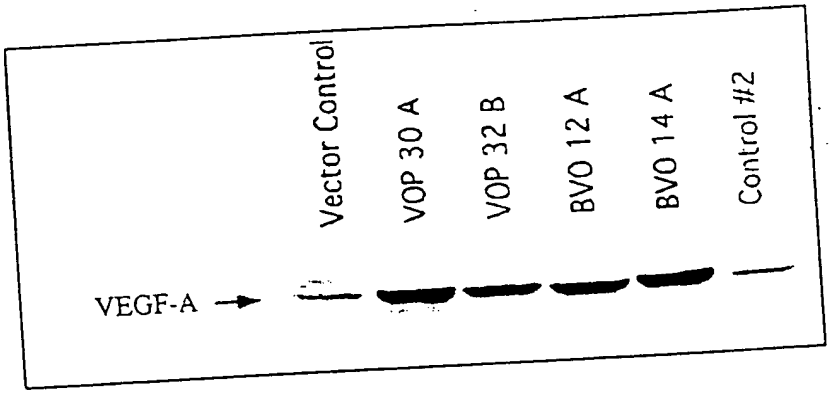

Vector Control
VOP 30 A
VOP 32 B
BVO 12 A
BVO 14 A
Control #2
VEGF-A

FIG 2

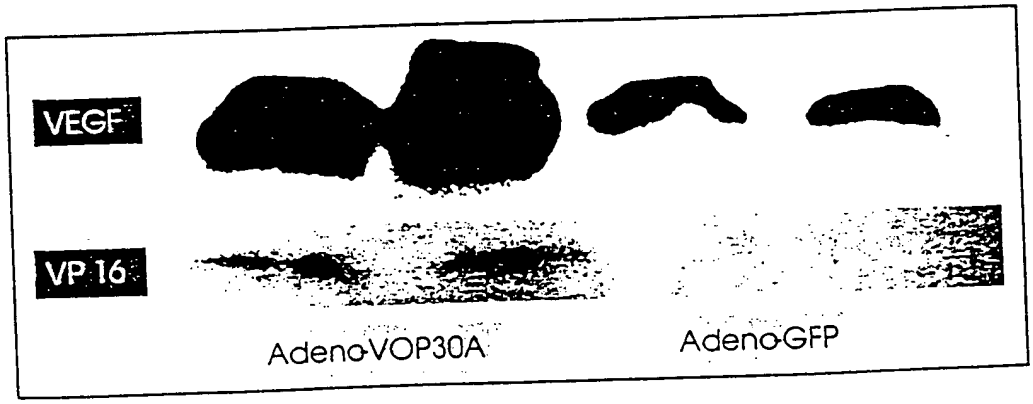


FIG3

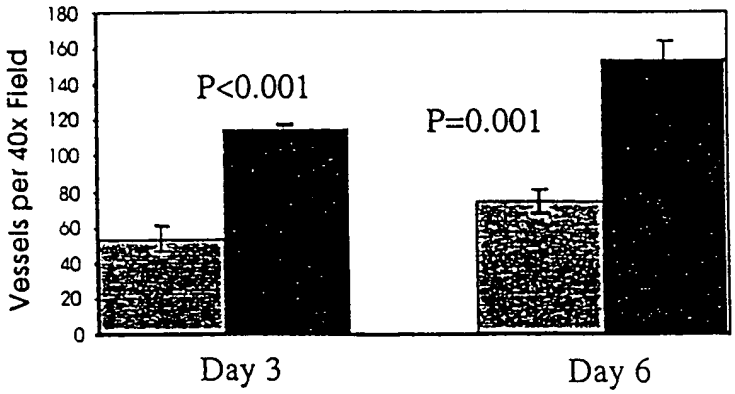

Vessels per 40x Field
180
160
140
120
100
80
60
40
20
0
P<0.001
P=0.001
Day 3
Day 6

FIG 4

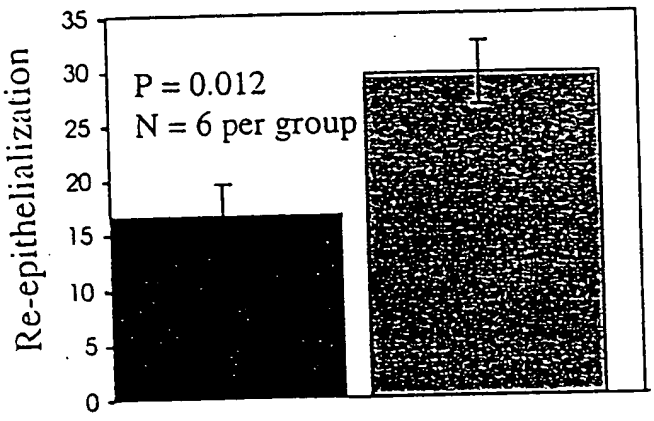


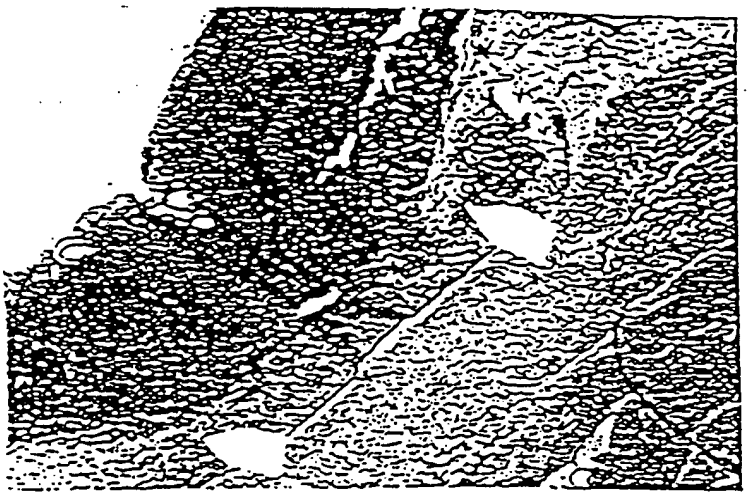
Fig. 5

Control

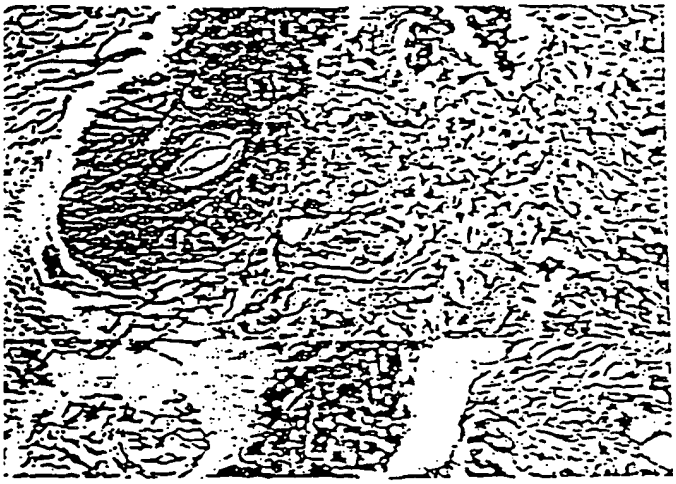
+ ZrP

A.

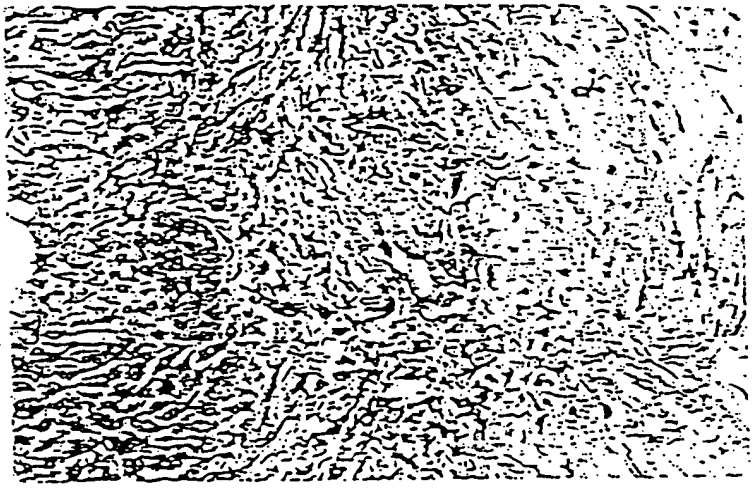
B.



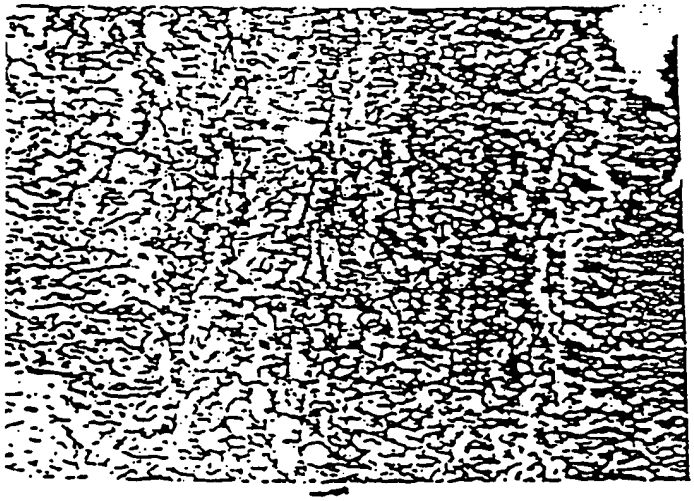
C.



D.



E.



F.

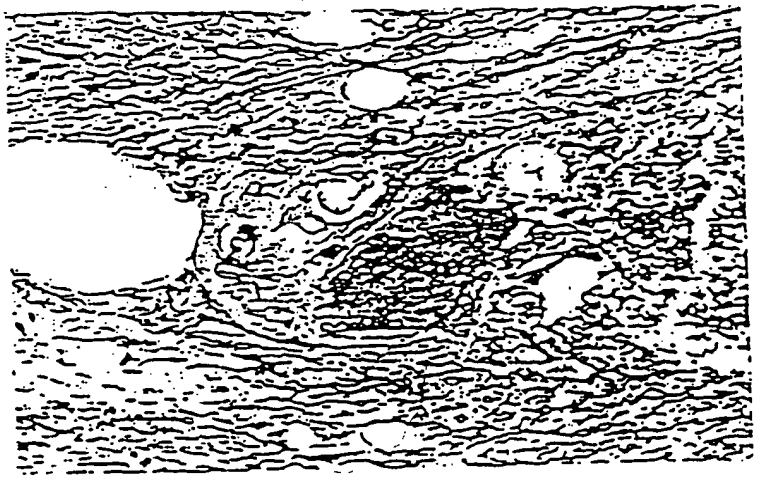


FIG 6

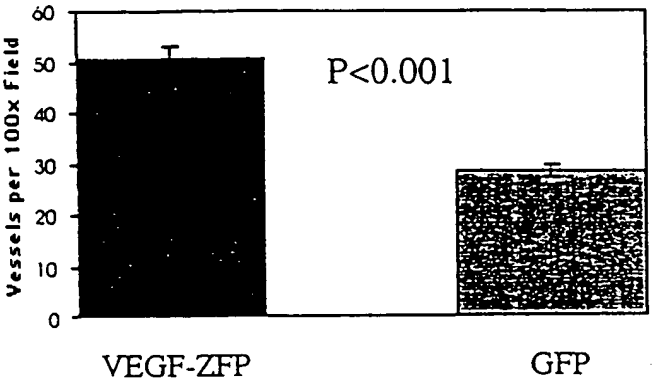


FIG 7

EXHIBIT C

Exhibit C: Experimental Protocols

A. Construction of Adenovirus Vectors

Recombinant E1A/E1B deleted adenovirus vectors encoding zinc finger protein (ZFP) cDNAs were produced using the Ad-Easy system (T.-C. He, *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95: 2509-2514). Briefly, cDNAs encoding VEGF regulating ZFPs were subcloned into the pAd-Track-CMV shuttle vector. This vector contains two separate CMV promoters, one to direct expression of inserted cDNAs and the other to direct expression of the transduction marker green fluorescent protein (GFP). In this shuttle vector, both the green fluorescent protein and the cDNA expression cassettes are flanked by genomic adenovirus-5 sequences, allowing recombination with genomic adenovirus-5 DNA (T.-C. He, *et al.* (1998)). More specifically, ZFP-encoding inserts from pcDNA MVG (a plasmid encoding the zinc finger binding domain VG10A/8A) and three-finger constructs pCV VOP 30A and pCV VOP 32B were cut by restriction digestion with EcoRI / HindIII and sub-cloned into shuttle plasmid pAdTrack-CMV, linearized with PmeI.

Each construct was co-electroporated along with the adenovirus-5 genomic vector pAd-Easy-1 into electrocompetent BJ5183 *E. coli* bacteria. Adenovirus-5 genomic DNA clones encoding the VEGF regulating ZFPs and deficient for the essential adenovirus E1A/E1B gene region were obtained by recombination between the ZFP encoding pAd-Track-CMV shuttle vectors and pAd-Easy-1 in *E. coli.* Kanamycin resistant colonies were screened for recombinants by restriction mapping, and clones with the appropriate restriction pattern were transfected into human embryonic kidney 293 cells (HEK 293's) using the Lipofectamine reagent. HEK 293's contain the E1A/E1B genes and provide these essential proteins in trans to allow production and propagation of the replication defective E1A/E1B deficient adenovirus recombinants. These recombinants do not propagate in normal mammalian cells due to the lack of E1A/E1B gene functions. Adenovirus stocks were expanded in 293 cells, purified by CsCl ultracentrifugation, desalted by column purification or dialysis against phosphate buffered saline, and titered on HEK 293 monolayers as described (see, *e.g.*, Giordano, *et al.* (1996) *Nat. Med.* 2:534-9; and Giordano *et al.* (1997) *Circulation* 96:400-3). Adenovirus stocks were stored in 20% glycerol at –80°C.

B. Induction of the VEGF-A gene in Skeletal and Cardiac Muscle by VEGF-ZFPs

1. Plasmid Injection Studies The ability of a number of different VEGF-A regulating ZFPs to induce expression of the endogenous VEGF-A gene was tested *in vivo* in rat skeletal muscle. Initial studies were carried out using plasmid DNA expression vectors encoding either a VEGF-A regulating ZFP fusion protein or a control protein containing the same peptide sequences absent the DNA binding domain. A commercially available plasmid in which gene expression is directed by the CMV promoter (pCDNA-3; Clontech) was used as the backbone for construction of the ZFP encoding plasmids.

Fifty μg of purified plasmid DNA encoding a VEGF-A regulating ZFP was diluted into 50 μl of phosphate buffered saline (PBS) and aspirated into a 1 cc syringe. For experiments in rats, a small incision was made in the skin overlying the adductor

muscle of the hindlimb of a Sprague-Dawley rat (or a CD-1 mouse), and the plasmid DNA was injected directly into the muscle through a 30 gauge needle. The site was marked by injecting a small amount of India ink at the point of plasmid DNA delivery. Fifty μg of the control plasmid was injected into the contralateral hindlimb adductor muscle using the same methods. At day 3 or 6 after plasmid delivery, animals were sacrificed and skeletal muscle from the plasmid injection sites was harvested using a 5 mm diameter punch bioptome. The muscle tissue was rinsed in ice-cold PBS and rapidly frozen in liquid nitrogen and used for subsequent VEGF-A protein expression analysis by Western blotting.

ZFP-directed VEGF-A protein expression was also evaluated in heart muscle using a similar approach. Briefly, in anesthetized and ventilated CD-1 mice, the heart was exposed by left lateral thoracotomy. Fifty μg of plasmid DNA encoding either a VEGF-ZFP or the control peptide was injected in a 50 μl volume into the apex of the heart. Injection was documented by blanching of the injection site. The chest was closed and the animal allowed to recover. Three days after injection the animals were sacrificed and the heart removed. The apex was clipped, rinsed in ice cold PBS, rapidly frozen in liquid nitrogen and used for subsequent VEGF-A protein expression analysis by Western blotting. Similar techniques can be used with rats.

2. Adenovirus Injection Studies. Using methodology similar to that used for plasmid DNA injection, VEGF-ZFP encoding or control (green fluorescent protein encoding) adenovirus vectors were injected into the hindlimb adductor muscle of CD-1 mice. Approximately 5 x 10^8 pfu of VEGF-ZFP encoding adenovirus was injected in a 50 μl volume of PBS into the adductor muscle using a 1cc syringe and a 30 gauge needle, as above. The contralateral adductor muscle was injected with a green fluorescent protein encoding control adenovirus. Three or six days after adenovirus injection the skeletal muscle injection site was harvested as above for Western blot analysis of VEGF-A protein expression. For those VEGF-ZFPs that were designed to bind to DNA sequences in both the murine and rat VEGF-A genes (VOP 30A, VOP 32B), adenovirus injections were also performed in the rat hindlimb adductor muscle. These studies were carried out essentially as above, although the volume of the injectate was increased to 100 μl. Related experiments were also conducted with plasmids in the same volume.

C. Western Blot Analysis

For Western Blot analysis of ZFP induced VEGF expression, animals were sacrificed by euthanasia on the 3rd day after injection. Skeletal muscles around the injection sites were carefully removed and homogenized at 4°C in lysis buffer containing Tris-HCl 50 mM (pH 8.0), NaCl 150 mM, SDS 0.1%, NP-40 1%, Na Desoxycholate (0.5%) and proteinase inhibitors. After a ten-minute centrifugation (10,000g at 4°C), the protein-containing supernatant fraction was collected, a small part of each sample was used to determine protein concentration (BioRad) and samples were diluted (1:1) with 2× loading buffer containing 50 mM DTT, and denatured by boiling 5 min. Samples containing equal amounts of total protein were loaded on and separated by electrophoresis through 12% SDS-PAGE gels (Tris-Glycine, Novex). Proteins in the gel were transferred to nitrocellulose membranes and the membranes were exposed to mouse monoclonal anti-VEGF antibody (RDI) to assess the level of VEGF expression, rabbit

polyclonal anti-VP16 antibody (Clontech) to determine expression of ZFP-VP16 fusion protein (and, hence, the amount of ZFP present), and mouse monoclonal anti-muscle specific actin antibody (NCL) as an internal standard. After several washes in TBST, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1h at room temperature, followed by TBST washes, and developed with an enhanced chemiluminescent substrate for detection of HRP (Pierce).

D. Murine Ear Angiogenesis Assay

1. Injection Methods To test the ability of the designed VEGF-ZFPs to induce angiogenesis *in vivo*, we evaluated vascularization in the murine ear after injection of adenovirus vectors encoding either a VEGF-regulating ZFP or a control peptide (green fluorescent protein). See, *e.g.*, Pettersson, A., *et al.* (2000) *Laboratory Investigation* 80:99-115. Briefly, using a 1cc syringe and a 30 gauge needle, ~3 x 10^8 pfu of VEGF-ZFP encoding adenovirus was injected subcutaneously into the mouse ear in a volume of ~25 µl PBS. The contralateral ear was similarly injected with an equal amount of control (green fluorescent protein)-encoding adenovirus. Three and six days after injection, the ears were visually inspected and digital photographs obtained using the same settings at the same distances. The animals were sacrificed, the ears harvested and fresh frozen in OTC for immunohistochemical analysis of angiogenesis.

2. Vessel Counts. Vascularization in the mouse ear was evaluated on fresh frozen tissue sections using an anti-lectin antibody (Vector Laboratories, Burlingame, CA) that is specific for endothelial cells (see, *e.g.*, Christie, K.N. and Thomson, C. (1989) *J. Histochem. Cytochem.* 37:1303-1304). Briefly, frozen sections were fixed with acetone, rinsed with PBS, and incubated for 2 hours with the anti-lectin antibody. Following three sequential wash steps, the sections were incubated for 2 hours with a secondary antibody linked to alkaline phosphatase and the slides were developed using a commercial alkaline phosphatase staining kit (Vecta Inc.). Vessel counts were determined on the basis of microscopic analysis of anti-lectin immunostaining by an observer blinded to the identity of the sections. Five separate 40x microscopic fields were evaluated per section, and the number of lectin stained vessels per field was averaged.

E. Wound Healing Assay To evaluate the ability of the VEGF-A regulating ZFPs to augment angiogenesis in a functional *in vivo* assay, we used a well-established model of cutaneous wound healing (see, *e.g.*, Swift, M.E., *et al.*, (1999) *Lab Invest.* 79:1479-87). Highly reproducible bilateral dorsal cutaneous wounds were created in CD-1 mice by excision of a 5 mm circle of skin using a 5 mm punch bioptome. Healing of these wounds involves production of granulation tissue, reepithelialization by ingrowth of keratinocytes and an angiogenic response. At the time of wound creation ~5 x 10^8 pfu of VEGF-ZFP encoding adenovirus was delivered to the wound site by topical application in a volume of 20 µl PBS. The contralateral wound site was treated with a control adenovirus encoding green fluorescent protein.

On day 5, the wound sites were excised whole, carefully bisected, formalin fixed and embedded in paraffin blocks. Multiple serial sections were obtained and used for histologic and immunohistologic analysis. Wound reepithelialization was analyzed on

hematoxylin and eosin stained sections evaluated under light microscopy with 4X and 40X objectives. Micrographic images were captured with a SPOT CCD camera and imported into Adobe Photoshop for analysis. Two parameters of reepithelialization were evaluated: a) the distance from the wound edge to the leading edge of keratinocyte ingrowth, and b) the distance between the leading edges of keratinocytes growing in from opposite sides of the wound. All measurements were made quantitatively using computer calipers, and all sections were evaluated by an observer blinded to the treatment.

Wound vessel counts were determined by immunostaining of wound sections using the endothelial cell specific anti-lectin antibody described above. Vessel counts from 5 separate microscopic fields were made per section (100x magnification) and the results per section averaged.